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RESEARCH PAPER

Ethylene-induced leaf senescence depends on age-related changes and *OLD* genes in *Arabidopsis*

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Abstract

Ethylene can only induce senescence in leaves that have reached a defined age. Thus, ethylene-induced senescence depends on age-related changes (ARCs) of individual leaves. The relationship between ethylene and age in the induction of leaf senescence was tested in *Arabidopsis* L*er*-0, Col-0, and Ws-0 accessions as well as in eight *old* (*onset of leaf death*) mutants, isolated from the L*er*-0 background. Plants with a constant final age of 24 d were exposed to ethylene for 3–16 d. The wild-type accessions showed a common response to the ethylene treatment. Increasing ethylene treatments of 3–12 d caused an increase in the number of yellow leaves. However, an ethylene exposure time of 16 d resulted in a decrease in the amount of yellowing. Thus, ethylene can both positively and negatively influence ARCs and the subsequent induction of leaf senescence, depending on the length of the treatment. The *old* mutants showed altered responses to the ethylene treatments. *old1* and *old11* were hypersensitive to ethylene in the triple response assay and a 12-d ethylene exposure resulted in a decrease in the amount of yellow leaves. The other six mutants did not show a decrease in yellow leaves with an ethylene treatment of 16 d. The results revealed that the effect of ethylene on the induction of senescence can be modified by at least eight genes.

Key words: Age-related changes, *Arabidopsis*, ethylene, leaf senescence, natural variation, *old* mutants.

Introduction

Senescence is the final developmental phase of a leaf which starts with nutrient salvage and ends with cell death. The first visible event during senescence is leaf yellowing, which typically starts at the leaf margins and progresses to the interior of the leaf blade (Quirino *et al*., 2000). The protein and RNA degradation parallels a loss in photosynthetic activity and the majority of the senescence processes have occurred by the time yellowing of the leaf can be seen (Buchanan-Wollaston *et al*., 2003). The degradation products are transported out of the leaves to other parts of the plant. In this sense, the senescing leaf continues to function as a source of nutrients to the whole plant, but at the expense of its own ability to survive (Bleecker and Patterson, 1997).

Senescence is under genetic control and requires differential expression of specific genes. Expression of photosynthesis-associated genes (PAGs) is down-regulated, while many other genes, designated as senescence-associated genes (SAGs), are up-regulated during senescence. Detailed studies on the SAG identities and their expression suggest a complex regulation of leaf senescence (Hensel *et al*., 1993; Buchanan-Wollaston, 1997; Gan and Amasino, 1997; Nam, 1997; Gan, 2003). In *Arabidopsis* the age of individual leaves plays a prominent role in determining leaf longevity (Hensel *et al*., 1993; Oh *et al*., 1997; Noodén and Penney, 2001; Jing *et al*., 2002), although floral initiation can influence plant longevity and thus whole-plant senescence (Levey and Wingler, 2005).

Lim *et al*. (2003) have suggested that a distinction should be made between the terms senescence and ageing: senescence refers to the process that leads to the death of the leaf, while ageing itself occurs throughout development,

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Abbreviations: ARCs, age-related changes; ARR, age-related resistance; PAGs, photosynthesis-associated genes; SAGs, senescence-associated genes.

from the initiation of a leaf primordium to senescence and death. Thus ageing determines when senescence starts, but not senescence itself. During ageing, age-related changes (ARCs) occur as a result of the differential regulation of developmental processes. Before senescence can be initiated, certain ARCs must have taken place in the leaf. An example of a process dependent on ARCs in *Arabidopsis* is age-related resistance (ARR) (Kus *et al*., 2002). The authors showed that plants become more resistant to *Pseudonomas syringae* as they age. It was suggested that the ability to accumulate SA is necessary for the ARR response and that SA may act as a signal for the production of the ARR-associated antimicrobial compound(s) and/or it may possess direct antibacterial activity against *P. syringae*. Examples of ARCs in other model-organisms are body movement and pharyngeal pumping in *Caenorhabditis elegans* (Huang *et al*., 2004) or accumulation of oxygen free radicals, protein conformational changes, and decline in chaperone functions in the brain of *Homo sapiens* (Drachman, 1997).

The components that control ARCs and thus ageing in plants are still unclear. One of the parameters for ageing is the photosynthetic capacity of the leaf. From the time of full leaf expansion, CO_2 fixation rates drop and the senescence programme is initiated (Thomas and Howarth, 2000), which indicates that metabolic flux shift may serve as a general signal for the induction of leaf senescence as implicated by the *ore4* mutation (Woo *et al*., 2002). During plant growth light dosage has an effect on ageing; high light intensity results in premature senescence when compared with growth under standard light intensities, while low light intensities delay the senescence process (Noodén *et al*., 1996). Many other stress-inducing conditions such as drought, darkness, ozone, and pathogen attack can hasten leaf senescence as well (Lim and Nam, 2005). Plant hormones, cytokinin and ethylene being the most conspicuous, are another group of plant endogenous components that play important roles in the regulation of the onset of senescence. While increasing cytokinin production could delay leaf senescence (Gan and Amasino, 1995; Ori *et al*., 1999), reducing endogenous cytokinin levels resulted in accelerated senescence (Masferrer *et al*., 2002). Recently, exciting advances have been achieved in dissecting the components involved in cytokinin signalling (Hutchison and Kieber, 2002; Hwang *et al*., 2002). Among the genes characterized, the receptor CKI1 and the response regulator ARR2 appear to be involved in regulating leaf senescence (Hwang and Sheen, 2001).

The plant hormone ethylene has long been seen as a key hormone in regulating the onset of leaf senescence. Zacarias and Reid (1990) reported that ethylene does promote senescence, but it is not an essential compound for the senescence syndrome induced by other factors (e.g. ABA). The role of ethylene in senescence has been demonstrated by several studies. Both ethylene-insensitive mutants *etr1-1* and *ein2* show increased leaf longevities (Grbić and Bleecker, 1995; Oh *et al*., 1997) and antisense suppression of the tomato ACC oxidase resulted in delayed leaf senescence (John *et al*., 1995). In these cases, however, senescence eventually begins and progresses normally. Exogenously applied ethylene induces premature leaf senescence in *Arabidopsis*. However, constitutive application of ethylene does not change the longevity of the leaves. Both *ctr1* mutants and *Arabidopsis* plants grown in the continuous presence of exogenous ethylene did not show premature senescence (Kieber *et al*., 1993; Grbić and Bleecker, 1995). Thus, ethylene is neither necessary nor sufficient for the occurrence of senescence. These studies suggest that ethylene does not directly regulate the onset of leaf senescence. It acts to modulate the timing of leaf senescence. Only when developmental changes controlled by leaf age are present can ethylene induce senescence (Hensel *et al*., 1993; Grbić and Bleecker, 1995; Jing *et al*., 2002).

In the present study, advantage was taken of natural variation among *Arabidopsis* accessions and the availability of *Arabidopsis onset of leaf death* (*old*) mutants to examine genetic loci that are involved in ethylene-induced senescence. The combined physiological and genetic studies used here showed that *Arabidopsis* accessions exhibited common and discrete senescence responses to exogenous ethylene. It was observed that ethylene displayed a dual function, both as an inducer and a repressor, in the induction of leaf senescence and that such a role of ethylene was differentially modulated by multiple genetic loci. These results lay out a basis for further molecular dissection of genes that regulate ethylene-induced leaf senescence.

Materials and methods

Plant material and growth conditions

Arabidopsis accessions Landsberg *erecta* (L*er*-0), Columbia (Col-0), and Wassilewskija (Ws-0) were used in this study. Plants were grown in a growth chamber at 21 °C and 65% relative humidity with a day length of 16 h. The light intensity was set at 120 μmol cm^{-2} s^{-1}. An organic-rich soil (TULIP PROFI No.4, BOGRO B.V., Hardenberg, The Netherlands) was used for the experiments described in Fig. 1. For the other experiments a γ-ray irradiated soil was used (Hortimea Groep, Elst, The Netherlands).

Plants for ethylene exposure were treated in a flow-through chamber at 20 °C and a humidity of 40% under continuous illumination. The ethylene dosage was set at 10 μl l^{-1} since it has been shown that a dose ranging from 1–100 μl l^{-1} was sufficient to generate similar effects on several ethylene responses, including leaf yellowing (Chen and Bleecker, 1995; H-C Jing, unpublished data).

For germination studies, seeds were surface-sterilized by soaking in 20% bleach for 5 min after which they were washed twice with sterile water. The sterilized seeds were plated on Murashige and Skoog medium containing 0.8% agar. The plates were stored at 4 °C for 4 d after which they were transferred to a growth chamber at 21 °C and 16 h of light. For the triple-response assay 10 μM filter-sterilized

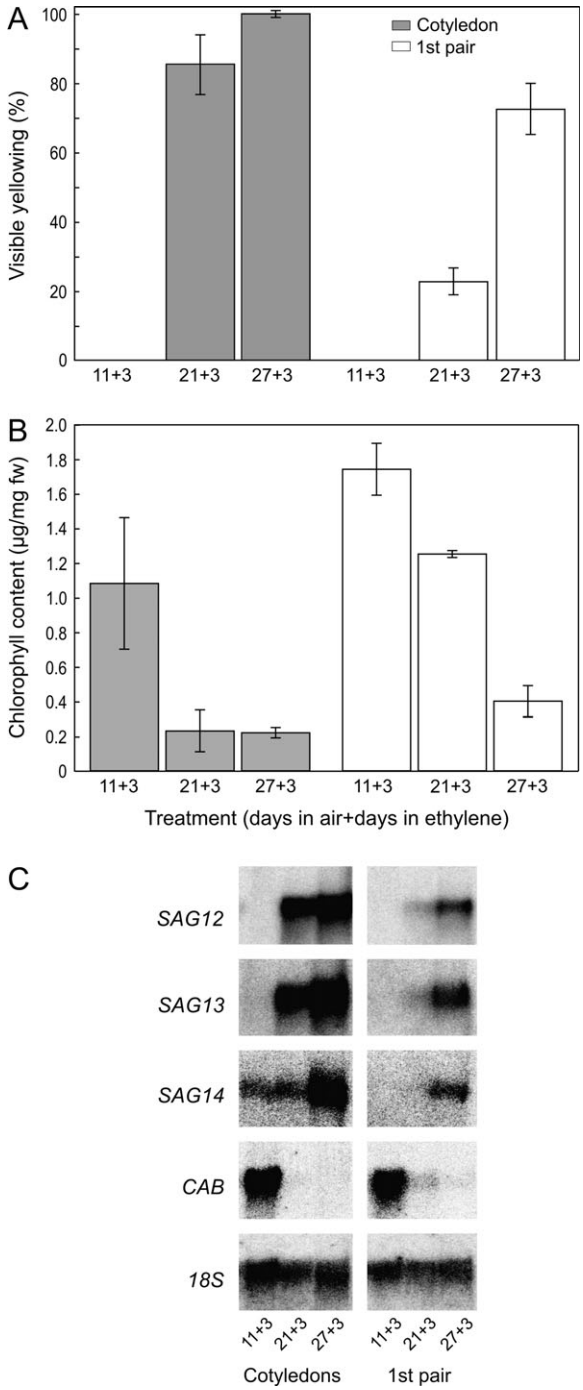

Fig. 1. Comparison of ethylene-induced senescence in cotyledons and rosette leaves of L*er*-0. Plants were grown first in air for 11, 21, or 27 d, and then exposed to ethylene for 3 d. The visible yellowing (A) of cotyledons and the first pair of rosette leaves was subsequently scored as the percentage of yellow cotyledons or rosette leaves versus the total number of cotyledons or rosette leaves, respectively. The chlorophyll contents (B) and steady-state mRNA levels (C) of several senescence-associated genes were subsequently analysed. For each time point, the observations on visible yellowing were on three sets of 50 plants and the results for chlorophyll is shown as mean ±SD of four replicates. Total RNA was isolated from leaf samples of approximately 100 plants. Five µg total RNA was used for northern blotting. The membrane was sequentially hybridized with cDNA probes of the indicated SAGs, CAB, and rRNA.

ACC was added to the plates. Seedlings were allowed to grow for 5 d in the dark before analysis.

Genetic analyses of the old *mutants*

Generation of M_1 and M_2 seeds and mutant screening were done as described by Jing *et al*. (2002). In this study, eight *old* mutants with early leaf senescence phenotypes were selected. Since the mutants were isolated from the L*er*-0 background, they were crossed to Col-0 plants for mapping. At least 100 F_2 plants with an early senescence phenotype were selected for DNA isolation using the SHORTY quick preparation method (http://www.hos.ufl.edu/meteng/HansonWebpagecontents/NucleicAcidIsolation.html#Arabidopsis%20Genomic%20DNA). The linkage analysis was performed by using a subset of simple sequence length polymorphism markers, making use of the Monsanto database (Jander *et al*., 2002). Both *old9* and *old11* are co-dominant traits and there was some overlap between the wild type and the heterozygous phenotypes of the mutants. In the F_2 populations of these two mutants backcrossed to wild type, sometimes a wild-type plant was found that had one yellow true leaf and occasionally a heterozygous mutant was found without any yellow true leaves following a 3 d ethylene treatment of 21-d-old plants. Thus, for the segregation analysis of *old9* and *old11*, any plant with at least one yellow true leaf was counted as a mutant. Plants with up to two yellow cotyledons were counted as wild types.

Observation of visible yellowing, chlorophyll content measurement, and northern blot analyses

Cotyledons or rosette leaves with over 5% yellow area of the leaf blade were judged as yellow as suggested by Lohman *et al*. (1994). However, in this study's experiments, yellowing could be initiated at the leaf tips, at the petiole side of the leaf, or in the middle of the leaf blade, which did not always resemble the wild-type developmental yellowing pattern of leaf senescence.

Chlorophyll was extracted in 80% (v/v) acetone overnight at 4 °C in darkness and quantified spectrophotometrically using the method of Inskeep and Bloom (1985). The RNA extraction and northern blot analyses was done as reported before by Jing *et al*. (2002). DNA from the following genes was used for the hybridization; *SAG12* (AT5G45890), *SAG13* (AT2G29350), and *SAG14* (AT5G20230) as described by Lohman *et al*. (1994). *CAB* gene expression was measured as described by Leutwiler *et al*. (1986).

Results

Physiological and molecular markers of leaf senescence correlate with visible yellowing

The effect of ethylene on age-dependent leaf senescence was determined for L*er*-0. L*er*-0 was grown for 11, 21, or 27 d after germination (DAG) and subsequently treated with ethylene for 3 d. The senescence syndrome was studied using morphological, physiological, and molecular markers (Fig. 1). Plants grown for 14, 24, or 30 d in air did not show any signs of visible yellowing, even though in the cotyledons the chlorophyll content had decreased approximately 2-fold over time (Jing *et al*., 2002). Plants grown for 11 d in air and subsequently for 3 d in air supplemented with ethylene (11+3) did not show any signs of senescence either. However, those treated for 21+3 and 27+3 showed signs of visible yellowing. Concomitant with the

visible yellowing, the chlorophyll content of the plants was decreased after the 21+3 and the 27+3 treatments (Fig. 1B). The chlorophyll content was lower in cotyledons than in the first leaf pair after both the 21+3 and the 27+3 treatments. The drop in chlorophyll content correlated with a decrease in the mRNA levels of *CAB* (Chlorophyll *a/b* binding protein) in the 21+3 and 27+3 samples (Fig. 1C). Expression of *SAG12*, *SAG13*, and *SAG14* was up-regulated in the 21+3 and 27+3 samples compared with the 11+3 samples. Low *SAG14* expression, however, was detected in the green cotyledons of 11+3 samples. The results are in agreement with previous studies, demonstrating that ethylene-induced senescence is dependent on leaf age (Grbić and Bleecker, 1995; Jing *et al*., 2002).

Thus the effect of ethylene on inducing visible yellowing correlated well with changes in chlorophyll content and in the *SAG* mRNA levels. The senescence syndrome continued being examined by using visible yellowing as a parameter.

Arabidopsis *accessions exhibited similar and discrete responses to the induction of senescence by different ethylene exposure times*

The effect of ethylene exposure time on leaf senescence was assessed in the *Arabidopsis* accessions L*er*-0, Columbia (Col-0), and Wassilewskija (Ws-0). Plants were grown until 8, 12, 18, or 21 DAG under standard conditions and were further grown under continuous light and ethylene conditions until 24 DAG. Thus, the plants that were treated for the longest time with ethylene received the treatment at a younger developmental stage. Figure 2 shows that ethylene has major effects on plant growth and development; the longest ethylene treatment caused the plants to phenocopy the *ctr1-1* mutant. The effect of the treatments on leaf yellowing was determined and the results are shown in Fig. 3. The overall response was similar for the three accessions tested; an increase in the duration of the ethylene treatment from 3 d to 12 d caused an increase in the number of yellow leaves. Longer ethylene treatments of 16 d resulted in a decrease in the number of yellow leaves, when compared with the 12 d ethylene treatment. These results are consistent with the phenotype of the *ctr1-1* mutant, which does not show early leaf senescence and the observation that continuous ethylene exposure does not promote early senescence in L*er*-0 (Kieber *et al*., 1993). There were also differences in ethylene-induced senescence among the three accessions. Ws-0 plants exhibited the highest amount of yellow leaves. L*er*-0, furthermore, had a more pronounced senescence response than Col-0. Thus, the number of yellow leaves that can be induced by ethylene is dependent on the genetic background and the length of the ethylene treatment.

Prolonged ethylene treatments inhibited cell expansion but stimulated plant development, as judged by leaf emergence. The average total leaf numbers in plants with various ethylene treatments were compared and the results showed that plants that experienced a longer ethylene exposure time had more leaves (Table 1). The increase in leaf number did not seem to correlate with the number of senescing leaves, Col-0 had more leaves than Ws-0 and L*er*-0, but exhibited less visible yellowing. Thus, the effect of ethylene exposure time on leaf senescence is different from its effects on the inhibition of cell elongation and promotion of development.

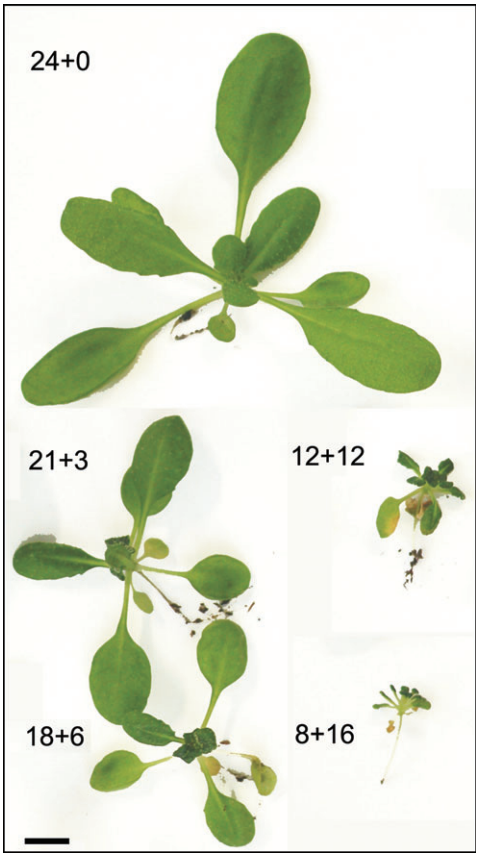

Fig. 2. Representative L*er*-0 plants after growth in air or after ethylene treatment. Plants were grown either in air for 24 d, or first in air for 21, 18, 12, or 8 d and then exposed to ethylene for 3, 6, 12, or 16 d, respectively. At the end of the treatment (24 d), representative plants were selected and photographed. The bar represents 0.5 cm.

Arabidopsis old *mutants reveal multiple genetic loci involved in the regulation of ethylene-induced leaf senescence*

As shown above, the genotype has an effect on the senescence response to ethylene. To address the genetic regulation of ethylene-induced leaf senescence further, use was made of previously isolated *old* mutants (Jing *et al*., 2002). The mutants were identified from an EMS mutagenized population of L*er*-0 seeds and show an early senescence phenotype before and/or after ethylene treatment (Fig. 4). The *old* mutants were selected and subgrouped into three different classes. The selected Class I

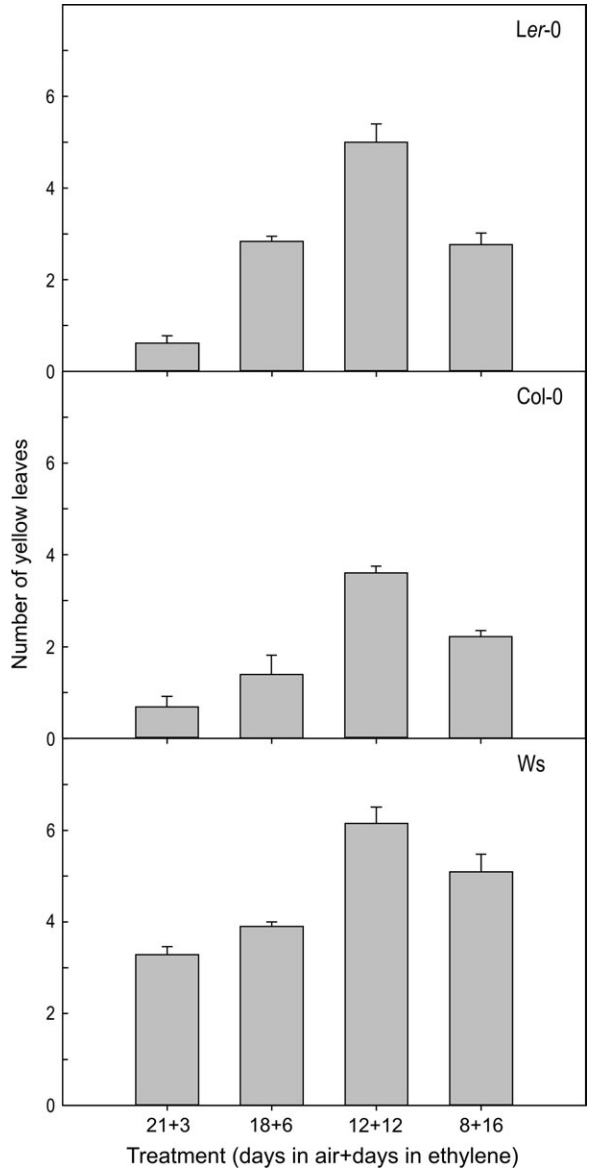

Fig. 3. Visible yellowing of 24-d-old wild-type plants, exposed to ethylene for various amounts of time. The number of yellow leaves of three *Arabidopsis* accessions exposed to different ethylene treatments is indicated. L*er*-0, Ws-0, and Col-0 plants were grown first in air for 21, 18, 12, or 8 d, and then exposed to ethylene for 3, 6, 12, or 16 d, respectively. The visible yellowing was subsequently scored and expressed as means ±SD of at least three replicates of 30 plants each. Plants that were grown for 24 d in air did not show any sign of senescence (not shown).

mutants comprises the formerly described mutant *old1* (Jing *et al*., 2002) and the mutants *old5* and *old14*. The mutants have early leaf senescence symptoms in air (compare Fig. 4A to C and E) and those symptoms are further enhanced by ethylene treatment (Fig. 4B, D, F). When grown in air, *old5* and *old14* did not differ markedly from the wild type, except for their early senescence phenotype which was observed after ~19 d. After 24 d of growth both mutants have 1–2 yellow leaves (results not shown). Air-grown Class II mutants were not different

Table 1. *Total leaf numbers of* Arabidopsis *accessions and* old *mutant lines after various ethylene treatments*

Plants were grown and treated with ethylene as described in Materials and methods. A leaf was scored when it emerged and was over 1 mm in size. Scoring was performed on 24-d-old plants at the end of the ethylene treatment. Approximately 30 plants were used for each line.

Lines	21+3	18+6	12+12	8+16
L*er*-0	12.7±0.2	13.3±0.2	15.1±0.2	15.1±0.4
Col-0	13.3±0.1	14.1±0.4	15.5±0.1	16.0±0.4
Ws-0	12.1±0.1	12.6±0.3	13.8±0.2	13.6±0.1
ctr1-1	11.8±0.1	11.8±0.2	13.0±0.5	13.0±0.1
old1	11.0±0.5	11.0±0.4	10.9±0.1	11.0±0.1
old5	12.6±0.2	13.3±0.1	14.4±0.3	13.9±0.2
old14	11.5±0.2	11.30±0.0	11.1±0.3	11.4±0.2
old9	12.7±0.3	12.7±0.1	13.7±0.1	14.5±0.1
old11	12.6±0.3	12.9±0.1	13.4±0.2	12.9±0.1
old13	12.1±0.3	12.4±0.3	14.0±0.2	13.8±0.3
old3	4.0±0	4.0±0	4.0±0	4.0±0
old12	11.5±0.0	11.5±0.1	12.1±0.0	12.3±0.4

from the wild type when grown under the standard conditions used here, but displayed enhanced senescence symptoms when treated with ethylene (Fig. 4G, H, I). In *old13*, ethylene-induced yellowing was associated with lesion formation. The *old12* mutant (Fig. 4J) and the previously described *old3* mutant (Jing *et al*., 2002) belong to the third class of mutants and have advanced senescence symptoms in air that cannot be further induced by ethylene treatment. The *old12* mutant not only displayed early senescence symptoms but also differed from the wild type in size, exemplified by reduced leaf expansion, shorter shoot length, and the formation of thicker siliques (data not shown).

Genetic analyses showed that the characterized *old* alleles segregated as single monogenic recessive traits except *old9* and *old11*, which segregated as conditional (ethylene-dependent) co-dominant traits (Table 2). Allelism tests between mutants within each class revealed that they belong to different complementation groups (data not shown). Genetic mapping placed the *old12* and *old9* mutant alleles on chromosome 2 at ~7 Mb and ~17 Mb, respectively; *old14* on chromosome 3 at ~15 Mb; and *old11*, *old5*, and *old13* on chromosome 5 at ~8 Mb, ~20 Mb, and 23 Mb, respectively. The authors are not aware of mutants with similar phenotypes in the vicinity of these mapped regions.

The response of the selected *old* mutants to several plant hormones and sugar was tested by germination assays. Since ethylene was used as the senescence inducer, the sensitivity of the mutants to ACC was tested by a triple-response assay (Table 3). Both *old11* and *old1* showed an extremely short hypocotyl. All other *old* mutants showed a wild-type response to ACC. Germination on glucose plates (Arenas-Huertero *et al*., 2000) showed that *old12* and *old1* are hypersensitive to glucose, moreover germination was completely inhibited by 4% glucose (data not shown).

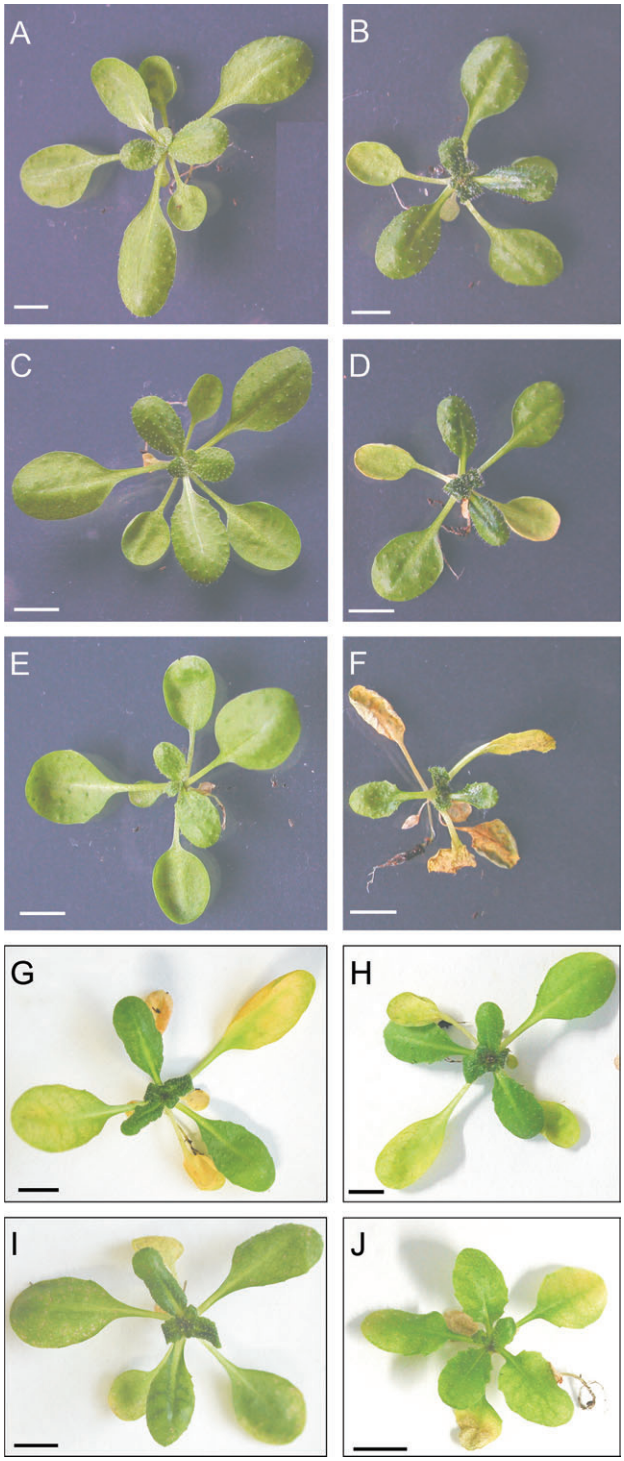

Fig. 4. Phenotype of *old* mutants. Representative 24-d-old L*er*-0 air-grown (A) and 21+3 ethylene-treated L*er*-0 (B). 24-d air-grown *old5* (C), *old14* (E), and *old12* (J). 21+3 ethylene-treated *old5* (D), *old14* (F), *old9* (G), *old11* (H), and *old13* (I).

The glucose phenotype was independent of osmolarity since all mutants developed like the wild type on mannitol. The response of the *old* mutants to ABA and jasmonic acid was not different from the wild type (data not shown).

Table 2. *Genetic segregation of* old *mutations*

Class	Male	Female	Generation	Wild type	Mutant	χ^{2c}
I[a]	*old5*	L*er*-0	F_1	31	0	
			F_2	151	64	2.61
	old14	L*er-0*	F_1	39	0	
			F_2	156	40	2.20
II[b]	*old9*	L*er*-0	F_1	23	25	
			F_2	18	73	1.32
	old11	L*er*-0	F_1	11	23	
			F_2	19	67	0.75
	old13	L*er*-0	F_1	52	0	
			F_2	180	79	4.18
III[a]	*old12*	L*er*-0	F_1	24	0	
			F_2	328	87	3.61

[a] The Class I and Class III mutants showed early senescence when grown in standard growth conditions. Thus, the scoring of phenotypes was performed before ethylene treatment and plants with clearly visible yellowing cotyledons and/or rosette leaves were scored as mutants.
[b] The segregation analysis for Class II mutants was performed after ethylene treatment. The phenotype scoring was carried out on 21-d-old plants treated with 10 μl l^{-1} ethylene for 3 d. The criteria were: wild-type plants with up to two yellow cotyledons, Class II mutants with at least 2 yellow cotyledons and 1 yellow leaf.
[c] All the χ^2 values were calculated for the 1:3 segregation ratios of mutant:wild type except in the case of *old9* and *old11*, where a 3:1 ratio of mutant:wild type was calculated.

Table 3. *Triple response assay of the wild-type* L*er-0 and the selected* old *mutants*

The average hypocotyl length after 5 d of growth on 10 μM ACC is compared. For each data point at least 40 seedlings were used.

Plant	MS medium (mm)	10 μM ACC medium (mm)
L*er*-0	6.89±0.19	3.25±0.14
old1	6.81±0.10	1.18±0.13
old5	7.01±0.28	3.05±0.19
old14	6.81±0.14	3.03±0.17
old9	6.74±0.12	3.07±0.05
old11	6.82±0.22	1.39±0.06
old13	6.99±0.20	3.17±0.03
old12	7.14±0.23	3.32±0.05

Thus, the selected *old* mutants show various senescence phenotypes that are caused by defects in independent genetic loci.

old *mutants and* ctr1-1 *have altered responses to varying ethylene-exposure lengths*

The *old* mutants and *ctr1-1* were exposed to ethylene for 3–16 d. Figure 5 shows that all the *old* mutants responded to the ethylene treatment in a different way than L*er*-0. The *old1* and *old11* mutants showed an enhanced response to long ethylene treatments: a maximum number of visible yellowing leaves occurred after the 18+6 treatment, instead of the 12+12 treatment in the L*er*-0 plants. A further increase in the length of ethylene treatment resulted in a reduced number of yellow leaves. Remarkably, *old1* had fewer yellow leaves than L*er*-0 after the 12+12 treatment

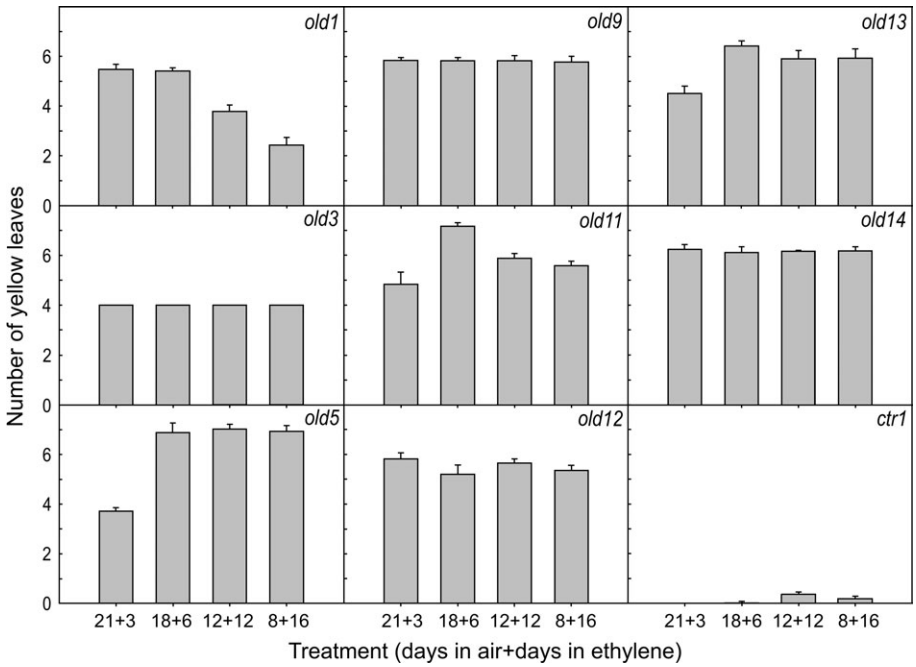

Fig. 5. Visible yellowing of 24-d-old *old* mutants and *ctr1-1*, exposed to ethylene for various amounts of time. The *old* mutants were grown first in air for 21, 18, 12, or 8 d, and then exposed to ethylene for 3, 6, 12, or 16 d. The visible yellowing was subsequently scored and expressed as means ±SD of at least three replicates of 30 plants each.

and so could be considered a delayed senescence mutant after long ethylene treatments. These data are consistent with the observation that *old1* and *old11* are hypersensitive to ethylene in the triple response. A similar number of yellow leaves, regardless of the length of the ethylene treatment, was observed in the *old3, old9*, *old12*, and *old14* mutants. Both *old3* and *old12* are Class III mutants, which were determined to senesce independently of ethylene, and the results shown here are consistent with that classification. Mutants of *old5* and *old13* had a constant number of yellow leaves when the ethylene treatment was longer than 3 d.

The average total leaf numbers of the mutants after various ethylene treatments were compared and the results showed that the *old1*, *old14*, and *old11* mutants responded differently to the ethylene treatments than the wild type (Table 1). In contrast to the wild type, these mutants did not show an increase in the rate of development, as judged by the emergence of new leaves. Nevertheless, in cases where the total number of leaves of the mutants was less than the wild type, the number of yellow leaves was higher. The one exception is the *old1* mutant, where after the 12+12 treatment, a reduced number of total leaves correlated with a reduced number of yellow leaves. The results suggest that it is unlikely that the differences observed in the senescence response are caused by different growth and developmental rates between the mutants and the wild type. Taken together, two mutants (*old1* and *old11*) became desensitized and showed fewer senescence symptoms upon extended long ethylene exposure, while the other six mutants show a similar response for every ethylene treatment.

Compared with the wild-type Col-0 plants, the *ctr1-1* mutant showed much lower numbers of yellow leaves in all the treatments tested, indicating that constitutively activating the ethylene signalling pathway through knocking-out *CTR1* has a pronounced effect in inhibiting the onset of leaf senescence. Interestingly, ethylene treatment did cause some leaf yellowing that was not observed in mutants that were not exposed to ethylene. Remarkably, the induction of leaf yellowing followed a similar response as the wild-type accessions: an increase in ethylene exposure time resulted in an increase in leaf yellowing up to 12 d of ethylene treatments. A further increase to 16 d caused a reduction in the number of yellow leaves.

Discussion

The effect of ethylene on the induction of leaf senescence was shown to be under the direct influence of age (Hensel *et al*., 1993; Grbić and Bleecker, 1995; Jing *et al*., 2002). The data presented here confirm these results as senescence was not induced in young cotyledons or leaves. The relationship between ARCs and ethylene-induced senescence was further assessed by treating plants of an identical end age with different ethylene exposure lengths. The three different *Arabidopsis* accessions showed a common

response to the different treatments implying a conserved perception mechanism of ethylene-inducible senescence. An increase in the length of the ethylene treatment, and thus treatment at a younger age, resulted in an increase in the amount of leaf yellowing. The longest treatment, however, caused a reduction in the amount of leaf yellowing. In particular, the *ctr1-1* mutants showed much lower numbers of yellow leaves compared with the wild-type plants, suggesting that constitutive activating ethylene signalling strongly inhibits the induction of leaf senescence. Thus, the effect of ethylene on leaf senescence relies on the exposure time. Since ethylene-induced senescence depends on ARCs, the results presented here suggest that ethylene treatment can influence ARCs. Depending on the length and start of the treatment, ethylene can stimulate, or suppress ARCs. Ethylene also caused the inhibition of cell expansion and the stimulation of plant development. In all accessions the longest ethylene treatment resulted in approximately two additional leaves. Here, the longest treatment did not cause a reduction in the stimulation of plant development. This suggests that the effect of the longest ethylene treatment on leaf yellowing is different from and independent of the effect on plant development. Besides the common response of the accessions to ethylene, natural variation was present and the Ws accession showed the strongest senescence symptoms after the ethylene treatments. These results confirm a previous study on natural variation in the regulation of leaf senescence by Levey and Wingler (2005). Natural variation has been observed for a variety of life history traits in *Arabidopsis* including the control of flowering time (Koornneef *et al*., 1998), disease resistance and tolerance (Kover and Schaal, 2002), and the control of cytosine methylation in the nucleolus organizer regions (Riddle and Richards, 2002). Remarkably, ethylene treatment of *ctr1-1* resulted in a senescence response that was similar to that of the three wild-type accessions. The *ctr1-1* mutant exhibits continuous activation of the ethylene signalling pathway and has a wild-type timing of senescence under standard growth conditions (Hensel *et al*., 1993). It was found here that, by applying exogenous ethylene, early senescence can be induced. Although *ctr1-1* loss-of-function mutants display a severe ethylene phenotype, these mutants remain ethylene responsive (Larsen and Chang, 2001). Quadruple loss-of-function mutants in the ethylene receptor family have a more severe phenotype than *ctr1-1* (Hua and Meyerowitz, 1998), suggesting that an alternative mechanism bypassing CTR1 in ethylene signalling exists in *Arabidopsis* (Cancel and Larsen, 2002). Since endogenous ethylene levels in *ctr1-1* mutants are equal or even lower than in wild-type plants (Ecker and Kieber, 1994) the observed senescence may be a direct cause of the applied exogenous ethylene which is signalled through an alternative ethylene pathway. Although the amount of yellowing was reduced in the *ctr1-1* mutants, the induction of senescence was age-dependent and ethylene exposure-time dependent. This suggests that the effect of ethylene on ARCs is maintained in *ctr1-1* mutants.

The results imply that ethylene stimulates ARCs in an ethylene treatment-duration dependent way. However, the relation between treatment length and induction of ARCs is not linear and there is an optimum treatment length that causes the strongest senescence response.

Previously, several *old* mutants were isolated that show an altered senescence phenotype before and/or after ethylene treatment (Jing *et al*., 2002). Eight *old* mutants were selected and the effect of different lengths of ethylene treatment on leaf yellowing was measured. Although all the mutants responded to the treatments in a different way from the wild type, there were two fundamentally different responses. Both *old1* and *old11* already showed a decrease in senescence with a shorter ethylene treatment than the wild type, suggesting that they may respond more strongly to the ethylene. Likewise, both *old1* and *old11* were found to be hypersensitive to ACC in the triple response assay. These results are consistent with the hypothesis that the mutants have an enhanced effect on ARCs as a result of a stronger response to ethylene. The second type of response was found in the other six mutants, where no decrease in leaf yellowing was found with the longest ethylene treatment. This suggests that the mutants may have lost the ability to suppress ARCs by long ethylene treatments. Interestingly, the six mutants fall into different classes. *old5* and *old14* represent Class I mutants, which already have an early senescence phenotype in air. The senescence phenotype of Class III mutants *old3* and *old12* is not dependent on ethylene and, as expected, a similar response was observed for every ethylene treatment applied. Only *old9* and *old13*, which are Class II mutants, are fully dependent on ethylene for their senescence phenotype.

In summary, it can be concluded that ethylene has an effect on processes that regulate ARCs in a treatment-duration dependent way. In eight *old* mutants this effect was altered, but the function of the *OLD* genes in the control of ARCs and ethylene-induced senescence is at present unclear. Future cloning and analysis of the *OLD* genes shall shed new light on the regulation of ethylene-induced leaf senescence and the role of ethylene on processes that control ARCs.

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References

Arenas-Huertero F, Arroyo A, Zhou Li, Sheen J, León P. 2000. Analysis of *Arabidopsis* glucose-insensitive mutants, *gin5* and

gin6, reveals a central role of the plant hormone ABA in the regulation of plant vegetative development by sugar. *Genes and Development* **14,** 2085–2096.

Bleecker AB, Patterson SE. 1997. Last exit: senescence, abscission, and meristem arrest in *Arabidopsis*. *The Plant Cell* **9,** 1169–1179.

Buchanan-Wollaston V. 1997. The molecular biology of leaf senescence. *Journal of Experimental Botany* **48,** 181–199.

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D. 2003. The molecular analysis of leaf senescence: a genomics approach. *Plant Biotechnology Journal* **1,** 3–22.

Cancel JD, Larsen PB. 2002. Loss-of-function mutations in the ethylene receptor *ETR1* cause enhanced sensitivity and exaggerated response to ethylene in *Arabidopsis*. *Plant Physiology* **129,** 1557–1567.

Chen QG, Bleecker AB. 1995. Analysis of ethylene signal-transduction kinetics associated with seedling-growth response and chitinase induction in wild-type and mutant *Arabidopsis*. *Plant Physiology* **108,** 597–607.

Drachman DA. 1997. Aging of the brain: a new frontier. *Annals of Neurology* **42,** 819–828.

Ecker JR, Kieber JJ. 1994. Constitutive triple response gene and mutations. *Agricultural Biotechnology Patents* 9284648.

Gan S. 2003. Mitotic and postmitotic senescence in plants. *Science of Aging Knowledge Environment 2003* **38,** re7.

Gan S, Amasino RM. 1995. Inhibition of leaf senescence by autoregulated production of cytokinin. *Science* **270,** 1986–1988.

Gan S, Amasino RM. 1997. Making sense of senescence (molecular genetic regulation and manipulation of leaf senescence). *Plant Physiology* **113,** 313–319.

Grbić V, Bleecker AB. 1995. Ethylene regulates the timing of leaf senescence in *Arabidopsis*. *The Plant Journal* **8,** 595–602.

Hensel LL, Grbić V, Baumgarten DA, Bleecker AB. 1993. Developmental and age-related processes that influence the longevity and senescence of photosynthetic tissues in *Arabidopsis*. *The Plant Cell* **5,** 553–564.

Hua J, Meyerowitz EM. 1998. Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. *Cell* **94,** 261–271.

Huang C, Xiong C, Kornfeld K. 2004. Measurements of age-related changes of physiological processes that predict lifespan of *Caenorhabditis elegans*. *Proceedings of the National Academy of Sciences, USA* **25,** 8084–8089.

Hutchison CE, Kieber JJ. 2002. Cytokinin signaling in *Arabidopsis*. *The Plant Cell* **14,** S47–S59.

Hwang I, Chen HC, Sheen J. 2002. Two-component signal transduction pathways in *Arabidopsis*. *Plant Physiology* **129,** 500–515.

Hwang I, Sheen J. 2001. Two-component circuitry in *Arabidopsis* cytokinin signal transduction. *Nature* **413,** 383–389.

Inskeep WP, Bloom PR. 1985. Extinction coefficients of chlorophyll *a* and *b* in *N*,*N*-dimethylformamide and 80% acetone. *Plant Physiology* **77,** 483–485.

Jander G, Norris SR, Rounsley SD, Bush DF, Levin IM, Last RL. 2002. *Arabidopsis* map-based cloning in the post-genome era. *Plant Physiology* **129,** 440–450.

Jing HC, Sturre MJ, Hille J, Dijkwel PP. 2002. *Arabidopsis* onset of leaf death mutants identify a regulatory pathway controlling leaf senescence. *The Plant Journal* **32,** 51–63.

John I, Drake R, Farell A, Cooper W, Lee P, Horton P, Grierson D. 1995. Delayed leaf senescence in ethylene-deficient ACC-oxidase antisense tomato plants: molecular and physiological analysis. *The Plant Journal* **7,** 483–490.

Kieber JJ, Rothenberg M, Roman G, Feldmann KA, Ecker JR. 1993. *CTR1*, a negative regulator of the ethylene response pathway in *Arabidopsis*, encodes a member of the raf family of protein kinases. *Cell* **72,** 427–441.

Koornneef M, Alonso-Blanco C, Blankestijn-de Vries H, Hanhart CJ, Peeters AJ. 1998. Genetic interactions among late-flowering mutants of *Arabidopsis*. *Genetics* **148,** 885–892.

Kover PX, Schaal BA. 2002. Genetic variation for disease resistance and tolerance among *Arabidopsis thaliana* accessions. *Proceedings of the National Academy of Sciences, USA* **99,** 11270–11274.

Kus JV, Zaton K, Sarkar R, Cameron RK. 2002. Age-related resistance in *Arabidopsis* is a developmentally regulated defense response to *Pseudomonas syringae*. *The Plant Cell* **14,** 479–490.

Larsen PB, Chang C. 2001. The *Arabidopsis eer1* mutant has enhanced ethylene responses in the hypocotyl and stem. *Plant Physiology* **125,** 1061–1073.

Levey S, Wingler A. 2005. Natural variation in the regulation of leaf senescence and relation to other traits in *Arabidopsis*. *Plant, Cell and Environment* **28,** 223–231.

Lim PO, Woo HR, Nam HG. 2003. Molecular genetics of leaf senescence in *Arabidopsis*. *Trends in Plant Science* **8,** 272–278.

Lim PO, Nam HG. 2005. The molecular and genetic control of leaf senescence and longevity in *Arabidopsis*. *Current Topics in Developmental Biology* **67,** 49–83.

Lohman KN, Gan SS, John MC, Amasino RM. 1994. Molecular analysis of natural leaf senescence in *Arabidopsis thaliana*. *Physiologia Plantarum* **92,** 322–328.

Leutwiler LS, Meyerowitz EM, Tobin EM. 1986. Structure and expression of three light-harvesting chlorophyll *a*/*b*-binding protein genes in *Arabidopsis thaliana*. *Nucleic Acids Research* **14,** 4051–4064.

Masferrer A, Arro M, Manzano D, Schaller H, Fernandez-Busquets X, Moncalean P, Fernandez B, Cunillera N, Boronat A, Ferrer A. 2002. Overexpression of *Arabidopsis thaliana* farnesyl diphosphate synthase (FPS1S) in transgenic *Arabidopsis* induces a cell death/senescence-like response and reduced cytokinin levels. *The Plant Journal* **30,** 123–132.

Nam HG. 1997. The molecular genetic analysis of leaf senescence. *Current Opinion in Biotechnology* **8,** 200–207.

Noodén LD, Hillsberg JW, Schneider MJ. 1996. Induction of leaf senescence in *Arabidopsis thaliana* by long days through a light-dosage effect. *Physiologia Plantarum* **96,** 491–495.

Noodén LD, Penney JP. 2001. Correlative controls of senescence and plant death in *Arabidopsis thaliana*. *Journal of Experimental Botany* **52,** 2151–2159.

Oh SA, Park JH, Lee GI, Paek KH, Park SK, Nam HG. 1997. Identification of three genetic loci controlling leaf senescence in *Arabidopsis thaliana*. *The Plant Journal* **12,** 527–535.

Ori N, Juarez MT, Jackson D, Yamaguchi J, Banowetz GM, Hake S. 1999. Leaf senescence is delayed in tobacco plants expressing the maize homeobox gene knotted1 under the control of a senescence-activated promoter. *The Plant Cell* **11,** 1073–1080.

Quirino BF, Noh YS, Himelblau E, Amasino RM. 2000. Molecular aspects of leaf senescence. *Trends in Plant Science* **5,** 278–282.

Riddle NC, Richards EJ. 2002. The control of natural variation in cytosine methylation in *Arabidopsis*. *Genetics* **162,** 355–363.

Thomas H, Howarth CJ. 2000. Five ways to stay green. *Journal of Experimental Botany* **51,** 329–337.

Woo HR, Goh CH, Park JH, Teyssendier de la Serve B, Kim JH, Park YI, Nam HG. 2002. Extended leaf longevity in the *ore4-1* mutant of *Arabidopsis* with a reduced expression of a plastid ribosomal protein gene. *The Plant Journal* **31,** 331–340.

Zacarias L, Reid MS. 1990. Role of growth regulators in the senescence of *Arabidopsis thaliana* leaves. *Physiologia Plantarum* **80,** 549–554.